JOURNAL OF BACTERIOLOGY, July 1998, p. 3692–3696 Vol. 180, No. 14
0021-9193/98/$04.00+0

Isolation and Characterization of Toluene-Sensitive Mutants from the Toluene-Resistant Bacterium *Pseudomonas putida* GM73

KWANG KIM, SUNGJIN LEE, KYUNGHEE LEE, AND DONGBIN LIM*

Department of Microbiology, Gyeongsang National University, Gazwadong 900, Chinju 660-701, Korea

Received 17 November 1997/Accepted 14 May 1998

To understand the mechanism underlying toluene resistance of a toluene-tolerant bacterium, *Pseudomonas putida* GM73, we carried out Tn*5* mutagenesis and isolated eight toluene-sensitive mutants. None of the mutants grew in the presence of 20% (vol/vol) toluene in growth medium but exhibited differential sensitivity to toluene. When wild-type cells were treated with toluene (1% [vol/vol]) for 5 min, about 2% of the cells could form colonies. In the mutants Ttg1, Ttg2, Ttg3, and Ttg8, the same treatment killed more than 99.9999% of cells (survival rate, $<10^{-6}$). In Ttg4, Ttg5, Ttg6, and Ttg7, about 0.02% of cells formed colonies. We cloned the Tn*5*-inserted genes, and the DNA sequence flanking Tn*5* was determined. From comparison with a sequence database, putative protein products encoded by *ttg* genes were identified as follows. Ttg1 and Ttg2 are ATP binding cassette (ABC) transporter homologs; Ttg3 is a periplasmic linker protein of a toluene efflux pump; both Ttg4 and Ttg7 are pyruvate dehydrogenase; Ttg5 is a dihydrolipoamide acetyltransferase; and Ttg7 is the negative regulator of the phosphate regulon. The sequences deduced from *ttg8* did not show a significant similarity to any DNA or proteins in sequence databases. Characterization of these mutants and identification of mutant genes suggested that active efflux mechanism and efficient repair of damaged membranes were important in toluene resistance.

Organic solvent partition preferentially in the cell membrane, and this accumulation causes expansion of the membrane and loss of membrane integrity (2, 25). This results in inhibition of membrane protein functions, disruption of proton motive force, and ensuing lysis and cell death. Organic solvents with a low log P_{ow} value (logarithm of the partition coefficient of the target compound in a mixture of *n*-octanol and water) are particularly toxic. Nevertheless, bacteria that are able to tolerate high concentrations of organic solvents in their culture medium do exist (1, 9, 10, 21). These bacteria have potential applications in bioremediation of contaminated sites and in bioconversion of water-insoluble compounds dissolved in appropriate solvents.

It was observed that some bacteria could adapt to high concentrations of toxic solvents (27). Alteration of the cell envelope structure was observed as the bacterium was exposed to organic solvents. Weber et al. observed an increase of *trans*-unsaturated fatty acid contents in cells grown with toluene (26). It was suggested that this isomerization of *cis*- into *trans*-unsaturated fatty acids plays an important role in solvent tolerance in bacteria (7, 22). To support this, a mutant lacking the *cis*→*trans* isomerization activity was sensitive to toluene (22). Pinkart et al. observed a modification of lipopolysaccharide and an increase in total fatty acids in solvent-treated cells in addition to the increase in *trans*-unsaturated fatty acid content (19). They suggested that these envelope modifications aid in bacterial survival at high concentrations of organic solvents. The presence of an active efflux system for toluene in solvent-resistant bacteria was also demonstrated (11), and this energy-dependent export system was shown to be important in toluene resistance (12). Studies by Ramos et al. showed that the increased cell membrane rigidity resulting from changes in fatty acid and phospholipid compositions, exclusion of toluene from the cell membrane, and removal of intracellular toluene by degradation all contribute to the toluene resistance of *Pseudomonas putida* DOT-T1 (22).

In this study, we took a molecular genetic approach in investigating genes functioning in the toluene tolerance of *P. putida* GM73, a field isolate resistant to high concentrations of toluene and other organic solvents. We carried out transposon mutagenesis with Tn*5* and isolated eight toluene-sensitive mutants. Characterization of these mutants and identification of mutant genes suggested that an active efflux mechanism and efficient repair of damaged membranes were important in the toluene resistance of *P. putida* GM73.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Escherichia coli* JM109 and *E. coli* JM83 were used as hosts for cloning and sequencing. *E. coli* C600(pGS9::Tn*5*) was used as a Tn*5* donor in transposon mutagenesis (5). *E. coli* HB101(pRK2013) was a helper in triparental mating (5, 23). *P. putida* ATCC 12633 and three toluene-resistant isolates, *P. putida* GM62, *P. putida* GM73, and *Pseudomonas* sp. strain GM80, isolated as described below, were grown in Luria-Bertani (LB) medium at 30°C. LB medium supplemented with 10 mM $MgCl_2$ (LBMg) was used when these bacteria were cultivated in the presence of toluene (10). To test toluene tolerance, cells were streaked on LBMg agar plate and plates were overlaid with toluene to a depth of at least 5 mm.

Isolation of toluene-resistant bacteria. Toluene-resistant bacteria were isolated from various soil samples collected from southern Korea. Drops of samples were directly inoculated into LBMg broth with 10% (vol/vol) toluene. The samples were incubated for 72 h at 30°C. In 3 out of 400 samples, bacterial growth was found. A single colony from each culture was isolated on LBMg agar plates overlaid with toluene. Colonies that appeared after 48 h of incubation at 30°C were purified and stored. For identification (24), the isolates were cultured on tryptic soy agar medium at 28°C for 48 h. Cells were harvested from the plates by scraping with a sterile glass loop and used for fatty acid methyl ester analysis. Saponification, methylation, and extraction were performed by using the procedures described in the MIDI manual (Microbial Identification, Inc.) (24).

Isolation of *P. putida* GM730. *P. putida* GM730, a mutant strain to which plasmids can be efficiently transferred by conjugation, was isolated as follows. *P. putida* GM73 was treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as described by Miller (15). MNNG-treated cells were grown as a single pool to

* Corresponding author. Mailing address: Department of Microbiology, Gyeongsang National University, Gazwadong, Chinju 660-701, Korea. Phone: 82-591-751-5946. Fax: 82-591-759-0187. E-mail: dblim@nongae.gsnu.ac.kr.

an optical density at 600 nm (OD_{600}) of 0.8. One milliliter of culture was transferred to a microcentrifuge tube and centrifuged. Cells were washed twice with saline and resuspended in 300 μl of saline. *E. coli* C600(pLAFR3) (23) and *E. coli* HB101(pRK2013), a plasmid donor and a helper, respectively, were cultivated and washed with saline as described above. They were resuspended in 300 μl of saline. Triparental mating was carried out by placing 30 μl of each strain with a micropipette onto LB agar plates. The plates were dried and incubated at 30°C. After 8 h of incubation, cells were collected by scraping and transconjugants were selected on LB plates containing tetracycline (30 μl/ml) for selection of plasmid pLAFR3 and ampicillin (50 μl/ml) for counterselection. From transconjugants, strains lacking plasmid pLAFR3 were isolated by replica plating cells grown overnight without tetracycline. Plasmid-free tetracycline-sensitive cells were picked and tested for toluene resistance. By performing subsequent mating experiments, we found that plasmids can be efficiently transferred by conjugation to these mutants. One of the mutants, *P. putida* GM730, was chosen for transposon mutagenesis.

Transposon Tn*5* mutagenesis. *E. coli* C600(pGS9::Tn*5*) (5) and *P. putida* GM730 were grown to an OD_{600} of 0.8. They were washed and mated on an agar plate as described in the previous section. Transconjugants were selected on plates containing ampicillin (50 μl/ml) and kanamycin (60 μl/ml). Colonies were picked and replica plated. Toluene was overlaid onto one of the plates, and toluene-sensitive mutants were screened. Colonies which could not grow in the plates overlaid with toluene were purified, and their toluene sensitivity was reconfirmed. It was confirmed, by Southern hybridization with Tn*5* DNA fragment as a probe, that these toluene-sensitive cells were derived from *P. putida* GM730 by a single Tn*5* insertion.

Toluene sensitivity of *ttg* mutants. Five milliliters of LBMg medium was inoculated with 50 μl of overnight culture, and cells were grown at 30°C until an OD_{600} of ca. 0.6 was reached. Fifty microliters of culture was taken and plated on LBMg agar with serial dilution. To the remaining culture, 50 μl of toluene was added and the mixture was incubated with agitation for 5 min. Cells were plated on LB agar, and colonies were counted after 24 h of incubation. Survival rates were calculated from the number of colonies present before and after toluene treatment.

Identification of *ttg* genes. DNA was isolated from mutant strains, digested with restriction enzyme *Eco*RI, which did not cut Tn*5*, and ligated to plasmid vector pTZ19R (New England Biolab). Colonies that appeared on the plates containing both kanamycin and ampicillin were collected, and the DNA sequence flanking Tn*5* was determined by using a synthetic primer (5′-CATGGA AGTCAGATCCT-3′) complementary to the distal end of Tn*5*. The obtained sequence was translated; amino acid sequences inferred from each open reading frame which had been interrupted by Tn*5* were compared with protein sequences in the database by using BLAST, and the function of each *ttg* gene was deduced from the similarity of its product to known proteins.

RESULTS

Isolation and characterization of toluene-resistant bacteria. Three gram-negative rods were isolated by the enrichment procedure described in Materials and Methods, and two of them were identified as *P. putida* and one was identified as *Pseudomonas* sp. (24). These were called *P. putida* GM62, *P. putida* GM73, and *Pseudomonas* sp. strain GM80. All three strains could grow in medium containing high concentrations (50% [vol/vol]) of solvents whose log P_{ow} values are greater than that of toluene (log P_{ow} = 2.5) (i.e., octane, propylbenzene, cyclohexane, *p*-xylene, and styrene), but they did not grow in medium with benzene and butanol, whose log P_{ow} values are lower than that of toluene.

Isolation of toluene-sensitive mutants. To investigate the molecular mechanism of toluene resistance, we tried to isolate toluene-sensitive mutants generated by transposon insertion. We attempted to mate our toluene-resistant strains and a donor *E. coli* carrying Tn*5*, but all attempts were unsuccessful. We reasoned that there might be some kind of barrier to the conjugative transfer in our toluene-resistant strains. This barrier was destroyed by mutation as described in Materials and Methods. One of the mutants, *P. putida* GM730, was chosen, and its growth was compared with that of the parent strain. The two strains showed similar growth patterns with doubling times of 45 min (data not shown). Cells started to aggregate when the cell density reached an OD_{600} of ca. 5.0. The aggregation was stronger in the wild type, and this made it difficult to measure cell density accurately in stationary phase. Both strains grew much more slowly in the medium containing toluene (doubling time, ca. 10 h), and the maximum cell density was twofold higher in the medium without toluene than in the culture medium with toluene (data not shown).

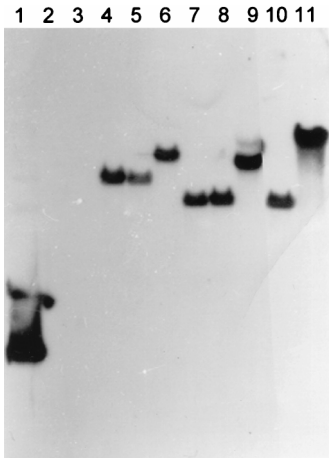

FIG. 1. Southern hybridization with Tn*5* DNA as a probe. Chromosomal DNA was isolated from each strain, digested with *Eco*RI, and electrophoresed on a 0.7% (wt/vol) agarose gel. DNA was blotted on a nylon membrane and hybridized with ^{32}P-labeled Tn*5* DNA as a probe. Lane 1, a plasmid containing Tn*5*; lanes 2 to 11, chromosomal DNA isolated from *P. putida* GM73 (lane 2), *P. putida* GM730 (lane 3), Ttg1 (lane 4), Ttg2 (lane 5), Ttg3 (lane 6), Ttg4 (lane 7), Ttg5 (lane 8), Ttg6 (lane 9), Ttg7 (lane 10), and Ttg8 (lane 11). Subsequent studies showed that Ttg1 and Ttg2 have mutations in the same gene and that genes *ttg4*, *ttg5*, and *ttg7* are closely linked (see text).

Transposon mutagenesis was carried out by conjugative transfer of Tn*5* from *E. coli* C600(pGS9::Tn*5*) to *P. putida* GM730, and toluene-sensitive mutants were screened by replica plating on the plates overlaid with toluene. From a screening of about 10,000 colonies, we isolated eight mutants which did not grow in the toluene-overlaid plates. These mutants should be affected in toluene tolerance genes, and they were called *ttg* mutants. Southern hybridization with a Tn*5* fragment as a probe showed a single hybridized band in all mutants, revealing that they were single transposon insertion mutants (Fig. 1).

Characterization of *ttg* mutants. All *ttg* mutants, especially Ttg4, Ttg5, and Ttg7, grew more slowly than the parent in LB medium (Fig. 2). Unlike the parental strain or other mutants, strains Ttg4, Ttg5, and Ttg7 did not grow in minimal medium with glucose, but they grew fine in medium with succinate. With 20% (vol/vol) toluene added to LBMg medium, no growth was observed for mutants Ttg1, Ttg2, Ttg3, Ttg4, Ttg5, Ttg7, and Ttg8 but Ttg6 grew after a long lag phase (15 h) (Fig. 2). In spite of this long lag phase, the growth rate of Ttg6 in medium with added toluene was similar to that of the wild type. Colonies isolated from the culture were no longer sensitive to toluene. Thus, we considered them revertants. All *ttg* mutants could grow in LBMg plate overlaid with *p*-xylene and styrene (13).

Sensitivity to toluene was examined by measuring the fraction of cells surviving after a short treatment with toluene. Cells cultivated in LBMg medium to log phase were treated with 1% toluene for 5 min. They were plated on LBMg agar medium with serial dilution, and the number of colonies that appeared was counted. For *P. putida* GM730, about 2% of cells survived after such treatment (Table 1). For mutants Ttg1, Ttg2, Ttg3, and Ttg8, no colonies were obtained, indicating that more than 99.9999% of the cells were killed. For mutants Ttg4, Ttg5, Ttg6, and Ttg7, about 0.02 to 0.05% of the cells survived. In the control experiment with toluene-sensitive *P.*

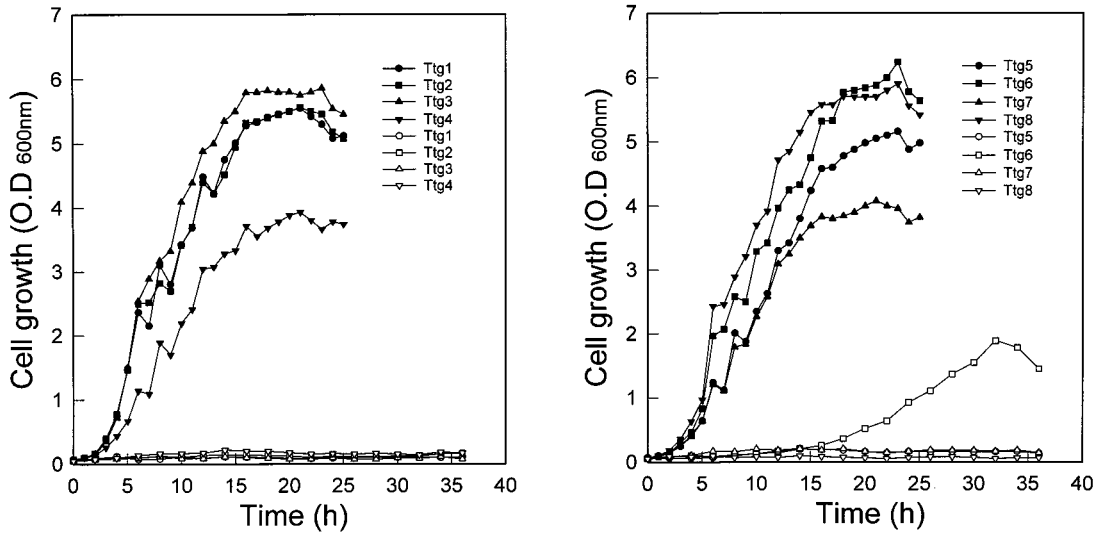

FIG. 2. Growth of toluene-sensitive mutants. Strains were cultivated at 30°C in LB medium (closed symbols) or LBMg medium with 20% toluene (open symbols).

putida ATCC 12633, no colonies appeared in the plates, indicating that more than 99.9999% of the cells were killed. It should be mentioned that 99 and 99.9999% of toluene-tolerant *P. putida* DOT-T1 and toluene-sensitive *P. putida* mt-2, respectively, were killed with similar treatments (22).

Identification of *ttg* genes. To elucidate possible functions of *ttg* genes, we cloned the Tn*5*-inserted genes, and the DNA sequence flanking the transposon was determined as described in Materials and Methods. The possible function of each *ttg* gene was inferred from a comparison of the translated amino acid sequence with protein sequences in a database.

Cloning and sequencing of *ttg1* and *ttg2* showed that their sequences at and around the Tn*5* insertion sites were identical but that the transposon orientations were opposite, showing that Tn*5* insertion occurred at the exact same positions. The amino acid sequence deduced from the DNA sequence of *ttg1* or *ttg2* had significant similarity to the sequences of a group of proteins known as transporters with an ATP binding cassette (ABC transporter) (Fig. 3) (4). The ABC transporter is a major system of bacteria participating in the export of a wide variety of substances, such as proteins, polysaccharides, antibiotics, and growth inhibitors (3). The amino acid sequence deduced from the *ttg3* sequence was almost the same as that of a periplasmic linker protein of the toluene efflux pump of *P. putida* S12 (12). Isken and de Bont showed that the energy-dependent efflux pump is important in toluene resistance (11), and recently Kieboom et al. cloned genes for this pump (12). They showed that the toluene efflux pump was composed of three proteins, an energy-dependent pump in the inner membrane (SrpB), a channel protein in the outer membrane (SrpC), and a periplasmic protein linking these two components (SrpA) (12). Thus, *ttg3* encodes the periplasmic linker protein of the toluene efflux pump of *P. putida* GM73.

As shown in Fig. 3, the deduced amino acids of both *ttg4* and *ttg7* have a high sequence homology with pyruvate dehydrogenase from various bacteria (8). From this observation, it was concluded that *ttg4* and *ttg7* are the genes encoding pyruvate dehydrogenase for *P. putida* GM73. We also found that a strong homology exists between the deduced amino acid sequence of *ttg5* and the sequence of dihydrolipoamide acetyltransferase, a component of the pyruvate dehydrogenase complex (20). Pyruvate dehydrogenase is a multienzyme complex comprising pyruvate dehydrogenase, dihydrolipoamide acetyltransferase, and lipoamide dehydrogenase. From these results, it is clear that the toluene-sensitive phenotype of three mutants (Ttg4, Ttg5, and Ttg7) is due to the lack of pyruvate dehydrogenase activity.

Sequence comparison of *ttg6* showed that it is *phoU* homolog (Fig. 3) (16). The gene product of *phoU* is a negative regulator of the *pho* regulon, and the *E. coli phoU* mutant constitutively produces alkaline phosphatase (16, 17). We found that alkaline phosphatase was constitutively expressed in the *ttg6* mutant (13), confirming that it is a *phoU* mutant of *P. putida* GM73. We do not know the function of *ttg8* since its deduced amino acid sequence did not show any significant similarity with any DNA or protein sequence in the database.

TABLE 1. Toluene sensitivity of mutants[a]

Strain	Colony count[b]
GM730	22,500
ATCC 12633[c]	0
Ttg1	0
Ttg2	0
Ttg3	0
Ttg4	372
Ttg5	190
Ttg6	528
Ttg7	284
Ttg8	0

[a] Cells were treated with toluene (1% [vol/vol]) for 5 min, and surviving cells were counted as described in Materials and Methods.

[b] Numbers are CFU normalized to 10^6 toluene-treated cells. Values are the averages of results from three independent experiments.

[c] *P. putida* ATCC 12633, a toluene-sensitive control.

DISCUSSION

Here we report the isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *P. putida* GM73. We isolated eight toluene-sensitive mutants, and sequence analysis showed that two were identical mutants and another two were mutants of the same gene. Therefore, we

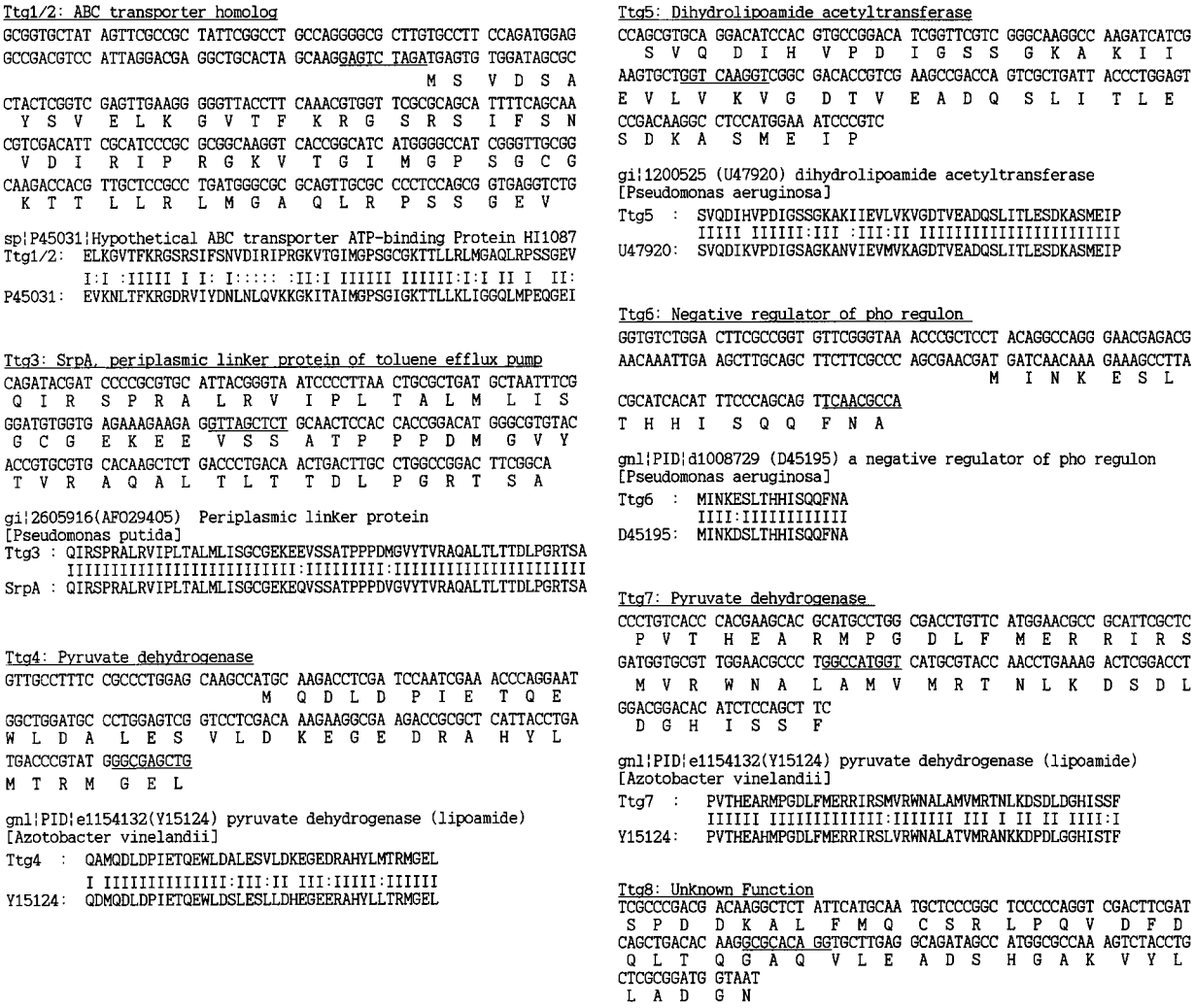

FIG. 3. Sequence analysis of *ttg* genes. The nucleotide sequence of each *ttg* gene flanking Tn*5* was determined, and the deduced amino acid sequence was compared with database sequences. Tn*5* insertion sites are underlined. The symbols I and : indicate identical and similar amino acids, respectively.

identified six genes which may play a role in toluene resistance of *P. putida* GM73.

Of eight mutants, three were found to be defective in the pyruvate dehydrogenase complex (Ttg4, Ttg5, and Ttg7). Pyruvate dehydrogenase catalyzes oxidative decarboxylation of pyruvate to acetyl coenzyme A (acetyl-CoA), which is a central enzyme in glucose metabolism. We found that Ttg4 and Ttg7 could not utilize glucose as a carbon, probably due to the lack of pyruvate dehydrogenase activity. It is reasonable to think that the lack of pyruvate dehydrogenase activity would lower the intracellular level of acetyl-CoA, a building block of fatty acid, and consequently this may affect membrane biosynthesis. Recently, Pinkart and White found an increase in phospholipid content and increased phospholipid turnover rate after exposure of bacteria to xylene (18). They suggest that solvent-resistant bacteria have a greater ability than solvent-sensitive bacteria to repair damaged membranes through efficient turnover and increased phospholipid biosynthesis. Because of the inadequate amount of acetyl-CoA in our mutants, the ability to repair damaged membranes is probably lower in our three mutants than in the wild type, and this may weaken the membrane rigidity and lower the permeability barrier. It should be noted that they could not grow in the medium containing 20% (vol/vol) toluene but that they still have some toluene tolerance as revealed by short-term treatment (Table 1).

Isken and de Bont and Ramos et al. reported that an energy-dependent efflux system is responsible for the resistance to toluene in *P. putida* S12 and DOT-T1 (11, 22). Recently the genes for the efflux system were cloned, and the efflux system was found to be a three-component pump with a striking resemblance to a multidrug efflux pump (12). Our sequence obtained from the *ttg3* gene is almost identical to that of *srpA*, a gene for the periplasmic linker protein of this efflux pump. Thus, strain Ttg3 is a mutant lacking the toluene efflux pump. The absolute lack of survival of Ttg3 cells after short-term treatment showed that this pump plays an important role in toluene resistance in our strain (Table 1).

The *ttg2* gene encodes a transporter protein containing an ATP-binding cassette (ABC transporter). The ABC transporter participates in the transportation of widely different substances (3). We found that Ttg2 is very sensitive to short-term treatment with toluene, suggesting the importance of this transporter in toluene resistance. At present, it is not clear whether this gene encodes a protein acting as a toluene pump.

There may exist two efflux pumps that participate in the toluene resistance of *P. putida* GM73. Alternatively, the gene may encode a transporter protein functioning in outer membrane synthesis, which is an important barrier to penetration by growth inhibitors (6).

Like mutant strains Ttg4, Ttg5, and Ttg7, some fraction of Ttg6 cells could survive after toluene treatment (Table 1). In Ttg6, alkaline phosphatase was constitutively expressed, indicating a phosphate deficiency in the cells. It is not clear whether the sensitivity to toluene is a direct effect of the phosphate deficiency in the cells or is an effect of physiological changes caused by the phosphate deficiency. When an outer membrane protein profile of the Ttg6 mutant was compared with that of the wild type, we found that a 44-kDa protein was overproduced in Ttg6 (13). It is possible that this 44-kDa protein forms an outer membrane channel for toluene, and its overexpression could result in the lower membrane permeability barrier observed in Ttg6, although Li et al. proposed that the 38-kDa protein OprF was a channel for toluene in *P. aeruginosa* (14). Alternatively, phosphate deficiency may affect phospholipid synthesis and thus alter membrane structure. This may lower the permeability barrier. The diffusion rate of ethidium bromide through the membrane into cytoplasm as measured with a fluorometer was found to be much greater in Ttg6 than in the wild type (13), suggesting that the permeability barrier of the membrane was lowered.

Since the Ttg8 mutant is very sensitive to toluene, as shown in Table 1, the mutated gene should encode a protein that plays an important role in toluene tolerance. Sequence analysis did not show a significant similarity between the deduced amino acid sequence of *ttg8* and any protein sequence in the database. Ramos et al. reported that a mutant lacking the *trans* isomers of the unsaturated C16:1 and C18:1 vaccenic fatty acids was sensitive to toluene, but it is not clear whether *ttg8* encodes *cis-trans* isomerase or not.

On the basis of these results, we are beginning to understand the general mechanism of toluene tolerance of *P. putida* GM73. Our analysis of Ttg mutants and other studies showed that three factors are important in the toluene resistance of *P. putida* GM73, namely, an active efflux pump(s), permeability barriers, and efficient repair of membrane damaged by solvent.

ACKNOWLEDGMENT

This work was supported by KOSEF 971-0502-010-2 from the Korea Science and Engineering Foundation.

REFERENCES

1. **Aono, R., M. Ito, A. Inoue, and K. Horikosh.** 1992. Isolation of novel toluene-tolerant strain of *Pseudomonas aeruginosa*. Biosci. Biotechnol. Biochem. **56:**145–146.
2. **Cruden, D. L., J. H. Walfram, R. D. Rogers, and D. T. Gibson.** 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl. Environ. Microbiol. **58:** 2723–2729.
3. **Faith, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
4. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, T.-F. Tomb, J. B. A. Dougherty, J. M. Merrick, K. Mckenney, G. Sutton, W. Fitzhugh, C. A. Fields, J. D. Gocayne, J. D. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. Mcdonald, K. Y. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
5. **Fredrickson, J. K., D. F. Bezdicek, F. J. Brockman, and S. W. Li.** 1988. Enumeration of Tn*5* mutant bacteria in soil by using a most-probable-number–DNA hybridization procedure and antibiotic resistance. Appl. Environ. Microbiol. **54:**446–453.
6. **Hancock, R. E. W.** 1997. The bacterial outer membrane as a drug barrier. Trends Microbiol. **5:**37–42.
7. **Heipier, H. J., F. J. Weber, J. Sikkema, H. Keweloh, and J. A. M. de Bont.** 1994. Mechanism of resistance of whole cells to toxic organic solvents. Trends Biotechnol. **12:**409–415.
8. **Hengeveld, A. F., A. H. Westphal, and A. de Kok.** 1997. Expression and characterization of the homodimeric E1 component of the *Azotobacter vinelandii* pyruvate dehydrogenase complex. Eur. J. Biochem. **250:**260–268.
9. **Inoue, A., and K. Horikoshi.** 1989. A *Pseudomonas* thrives in high concentration of toluene. Nature **338:**264–266.
10. **Inoue, A., M. Yamamoto, and K. Horikoshi.** 1991. *Pseudomonas putida* which can grow in the presence of toluene. Appl. Environ. Microbiol. **57:**1560–1562.
11. **Isken, S., and J. A. M. de Bont.** 1996. Active efflux of toluene in a solvent-resistant bacterium. J. Bacteriol. **178:**6056–6058.
12. **Kieboom, J., J. J. Dennis, J. A. M. de Bont, and G. J. Zylstra.** 1998. Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J. Biol. Chem. **273:**85–91.
13. **Kim, K., and D. Lim.** Unpublished data.
14. **Li, L., T. Komatsu, A. Inoue, and K. Horikosh.** 1995. A toluene-tolerant mutant of *Pseudomonas aeruginosa* lacking the outer membrane protein F. Biosci. Biotechnol. Biochem. **59:**2358–2359.
15. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Muda, M., N. N. Rao, and A. Torriani.** 1992. Role of *phoU* in phosphate transport and alkaline phosphatase regulation. J. Bacteriol. **174:**8057–8064.
17. **Nikata, T., Y. Sakai, K. Shibat, J. Kato, A. Kuroda, H. Ohtake.** 1996. Molecular analysis of the phosphate-specific transport (pst) operon of *Pseudomonas aeruginosa*. Mol. Gen. Genet. **250:**692–698.
18. **Pinkart, H. C., and D. C. White.** 1997. Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. J. Bacteriol. **179:**4219–4226.
19. **Pinkart, H. C., J. W. Wolfram, R. Rogers, and D. C. White.** 1996. Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. Appl. Environ. Microbiol. **62:**1129–1132.
20. **Rae, J. L., J. F. Cutfield, I. L. Lamont.** 1997. Sequences and expression of pyruvate dehydrogenase genes from *Pseudomonas aeruginosa*. J. Bacteriol. **179:**3561–3571.
21. **Ramos, J. L., E. Duque, M.-J. Huertas, and A. Haïdour.** 1995. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J. Bacteriol. **177:**3911–3916.
22. **Ramos, J. L., E. Duque, J.-J. Rodriguez-Herva, P. Godoy, and A. Fernandez-Barrero.** 1997. Mechanism for solvent tolerance in bacteria. J. Biol. Chem. **272:**3887–3890.
23. **Ronald, P. C., J. M. Salmeron, F. M. Carland, and B. J. Staskawicz.** 1992. The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J. Bacteriol. **174:**1604–1611.
24. **Sasser, M.** 1990. Technical note 102: tracking a strain using the Microbial Identification System. MIS Inc., North Newark, Del.
25. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1995. Mechanism of membrane toxicity of hydrocarbons. Microbiol. Rev. **59:**201–222.
26. **Weber, F. J., S. Isken, and J. A. M. de Bont.** 1994. Cis/trans isomerization of fatty acids as a defense mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiology **140:**2013–2017.
27. **Weber, F. J., L. P. Oojikaas, R. M. W. Schemen, S. Hartmans, and J. A. M. de Bont.** 1993. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. Appl. Environ. Microbiol. **59:**3502–3504.